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GaAs (904-Nm) Laser Radiation Does Not Affect Muscle Regeneration in Mouse Skeletal Muscle

Nuno M.L. Oliveira, PT,[1] Nivaldo A. Parizzotto, PhD, PT,[1] and Tania F. Salvini, PhD, PT[2]*

[1]Master Physical Therapy Program, Universidade Federal de São Carlos, 13565-905 São Carlos, SP, Brazil
[2]Department of Physical Therapy, Universidade Federal de São Carlos, 13565-905 São Carlos, SP, Brazil

Background and Objective: **We evaluated the effect of GaAs (904-nm) laser, applied directly to the skin of injured muscle, in muscle regeneration.**
Study Design/Material and Methods: **Muscle injury was induced in the *Tibialis anterior* (TA) muscle by ACL myotoxin (5 mg/kg). Two groups were irradiated with doses of 3 (n = 8) and 10 J/cm^2(n = 8). GaAs laser (power 1.5 mW, intensity 7.5 mW/cm^2, spot 0.2 cm^2) was applied daily for five days. Contralateral TA received a sham procedure.**
Results: **Similar morphological aspects were found in both laser irradiated and sham muscles. No differences were found in the muscle weight, but animals irradiated with 10 J/cm^2 showed a significant gain of body weight (P = 0.002).**
Conclusions: **Doses of 3 and 10 J/cm^2 of GaAs laser were not efficient to promote significant morphological changes in the regenerated skeletal muscle, but the dose of 10 J/cm^2 promoted significant gain of body weight. Lasers Surg. Med. 25:13–21, 1999.** **© 1999 Wiley-Liss, Inc.**

Key words: muscle injury; muscle regeneration; skeletal muscle; *Tibialis anterior;* ACL myotoxin

INTRODUCTION

The efficacy of low power laser therapy used in the treatment of soft tissues is controversial. The interaction of low energy laser radiation with biological systems is best established at the cellular level, but despite this known activity, the utility of the laser as a physical therapy agent remains polemical [1,2].

Although the process of muscle fiber regeneration is well studied [3] there are some questions that remain to be answered, especially concerning the effect of different physical agents frequently used to promote the regeneration process. Previous reports indicated that low power helium-neon (HeNe) promote skeletal muscle regeneration in both mammals and amphibians [4–8]. It was also described that muscle regeneration was equally promoted by single irradiation of gallium arsenide (GaAs) or HeNe lasers, however multiple

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N. M. L Oliveira is a Master student of the Physical Therapy Program at the Universidade Federal de São Carlos, and is the recipient of a Master Fellowship from CAPES/Brazil.

*Correspondence to: Tania F. Salvini, Departamento de Fisioterapia, Universidade Federal de São Carlos, 13565-905 São Carlos/SP, Brazil. E-mail: tania@power.ufscar.br

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irradiation of GaAs laser caused some pathological changes in the newly formed muscular structures [7].

Some studies have been developed to evaluate the effect of low-power energy GaAs laser in biological tissues, but its efficacy to stimulate cell proliferation and to promotes the regeneration process is also debatable in the literature. For example, GaAs laser did not have biostimulatory effects on fibroblasts and keratinocytes cultures [9], was not effective in the inflammatory function of human monocytes and endotelial cells in vitro [10], failed to stimulate the healing process of venous ulcers [11], healing of burns [12] and in the treatment of chondromalacia patellae [13] in humans, and was also not effective in the wound healing in rats [14]. However, some reports described that GaAs laser irradiation was effective to promote regeneration to the toad gastrocnemius [7], to increase the number and degranulation of mast cell in rats [15], and in the treatment of shoulder tendonitis [16], osteoarticular pain [17], and tennis elbow [18] in humans.

It is important to note that in several studies that showed a beneficial effect of low power lasers on the skeletal muscle regeneration, the muscles were directly irradiated [4–6]. This procedure cannot be used in a clinical situation in which the skin remains intact.

Recently, we studied the effect of different doses (2.6, 8.4, and 25 J/cm^2) of HeNe laser in the regeneration of *Tibialis anterior* (TA) of mouse (Amaral, Parizzotto, and Salvini, unpublished results). Laser irradiation was made directly to the intact skin of the injured muscle, once daily during five consecutive days, with the first application being made two hours after the induction of injury. Only the dose of 2.6 J/cm^2 promoted significant changes in the regeneration process of irradiated muscles, which showed an increase in both mitochondrial density, observed by succinate desidrogenase reaction, and muscle fiber area (3,280 ± 704 μm^2 vs. 2,110 ± 657 μm^2, $P = 0.02$), when it was compared with damaged but non-irradiated sham muscles. These results indicated that the effect of HeNe laser, in the muscle regeneration of mouse is dose dependent.

HeNe laser irradiation has a penetration depth of a few millimeters, whereas longer wavelength infrared radiation may penetrate several millimeters more. Therefore, it has been suggested that HeNe lasers are more effective for superficial wounds, and infrared lasers more effective for deeper conditions [1,2]. Thus, it would be interesting to evaluate the effect of GaAs on the regeneration of skeletal muscle using the same protocol developed in our laboratory to evaluate the effectiveness of HeNe laser on muscle regeneration (Amaral, Parizzotto, Salvini, unpublished results), because muscle fiber injuries may be located in both superficial and deep regions of the skeletal muscles and probably GaAs laser could have a better penetration in the muscle than HeNe laser. Also, it has been described that the transmission of light through tissue is highly wavelength specific and depends on spectral absorption in the molecules chromophores [19,20]. Consequently, possible differences between HeNe and GaAs lasers in the regeneration process of mouse muscle could be found.

The purpose of the experiments reported here was to determine the ability of indirect irradiation of GaAs laser, i.e., applied directly to the intact skin of injured muscle, to alter the regeneration process of mouse skeletal muscle.

MATERIALS AND METHODS

Animal Care

Sixteen male mouse (white Swiss), weighing 40.5 ± 2 g were used. They were randomly housed in two groups. Animals of each group (n = 8) were housed in a common standard plastic cage in an animal room with controlled environmental conditions (12 h dark/light cycle; temperature 22.5°C). They received standard food and had free access to food and water. All samples survived and were subsequently analyzed.

Muscle Injury

Under deep ethyl ether anesthesia animals were weighed and the skin of right and left TA muscles was shaved and cleaned. Muscle injury was induced in both muscles by one intramuscular injection of ACL myotoxin (5 mg/kg) applied in the middle region of the muscle.

TA muscle was chosen for this study because it is an accessible muscle and is easily located for the injection of ACL myotoxin and laser application. As previously described it possesses longitudinal fiber architecture and because its fibers extend ~ 70% of the muscle length, cross-sections taken from the muscle middle belly contain all fibers, avoiding sampling problems [21].

ACL Myotoxin

ACL myotoxin was obtained from Dr. Charlotte Ownby of the Department of Anatomy, Pa-

thology, and Pharmacology, Oklahoma State University, Stillwater, OK, USA. It was first isolated by Johnson and Ownby [22], and it was determined to be a Lys49 type II phospholipase A2 (PLA2) [23]. ACL myotoxin was purified from the crude venom of the Broad-Banded Copperhead (*Agkistrodon contortrix laticinctus*) as previously described [22]. Briefly, this consists of fractionation of crude venom by anion exchange chromatography followed by final purification using cation exchange chromatography.

GaAs Laser Irradiation

Before starting the experiments, the GaAs laser equipment used was previously calibrated in a laser power energy monitor (Ophir Optronics INC, Model 3A-P-DGX, Jerusalem, Israel)[1] at the Institute of Physic, Universidade de São Paulo, São Carlos, Brazil.

We used a pulsed GaAs laser of 904-nm wavelength, impulse duration of 200 ns, frequency of 1,000 Hz, peak power of 7 W, average power output of 1.5 mW, intensity of 7.5 mW/cm^2, and spot of 0.2 cm^2. The time of irradiation for each laser application was 2.2 minutes (group 3 J/cm^2) and 8 minutes (group 10 J/cm^2), and was automatically controlled by the laser equipment. Each group was once daily irradiated with 3 J/cm^2 (n = 8) or 10 J/cm^2 (n = 8) of GaAs laser. These doses were chosen because previous studies showed a beneficial effect of low power lasers on the regeneration process using doses among 1 and 4 J/cm^2 [1,2] and also because high doses have been described to stimulate [24] or to retard this process [25].

Before the laser application the animals were submitted to anesthesia with ethyl ether and maintained in a containment cage where their body and legs where fixed by adhesive tape. The knee joint was maintained in maximal extension and ankle joint was fixed in flexion (90°). Laser treatment was applied daily at the same time on five consecutive days, with the first application being made two hours after the induction of the injury. The laser was applied directly to the skin of the middle region of TA muscle, which was previously shaved and cleaned. The incidence angle of the beam laser was maintained perpendicular (90°) to the irradiation surface. This procedure was performed in a similar way on the contralateral TA muscle, but without the emission of radiation (sham group).

Histology and Histochemistry

Twenty one days after ACL myotoxin injection the animals were weighed under deep ethyl ether anesthesia. Right and left TA muscles were removed, weighed, immediately frozen in melting isopentane, and stored in a freezer at –56°C. Afterward, the animals were killed by overdose of anesthesia. Frozen muscles were cut (10 μm cross-sections) through the proximal to distal region using a cryostat (Microm, Germany). Alternate serial cross-sections were obtained in the proximal and middle regions of both muscles and stained with 1% Toluidine Blue/ 1% Borax or incubated for myofibrillar ATPase activity (mATPase) after alkali (alc-mATPase, pH 10.3) [26,27] or acid pre-incubations (ac-mATPase, pH 4.3) [28], Succinate Desidrogenase (SDH) [29], Acid Phosphatase [30] and Acetylcholinesterase (AChE) [31]. Toluidine Blue staining enables us to identify the morphological characteristics of muscle fibers and the signals of previous muscle injury such as the presence of split fibers and centralized nuclei. Myosin ATPase reaction is used for identification of the muscle type fibers. The type I fibers react deeply after acid pre-incubation (pH 4.3) and lightly after alkali pre-incubation (pH 10.3). The inverse occurs with the type II muscle fibers. Succinate desidrogenase allow to identify the muscle fibers with oxidative metabolism. Acid phosphatase reaction is the most accurate histochemical criterion of muscle cell necrosis or cell phagocitosis and is observed during the first days after muscle injury [32]. It was used here to confirm the regeneration stage of the muscle fibers. AChE activity allows the identification of the skeletal muscle endplate.

Although serial cross-sections submitted to mATPase reactions were utilized in this study to identify muscle type fibers I, II, and IIC, it is known that adult mammalian skeletal muscles express multiple isoforms of myosin heavy chain (MHC), which has been classified as slow type I, fast type IIA, and fast type IIB muscle fibers [33]. A new MHC was discovered in small mammals and classified as type IIX [34]. Type IIX MHC appears to be histochemically characterized by an aerobic oxidative capacity intermediate between that of the type IIB and that of type IIA [34,35]. Thus, throughout this study, type II fibers will be

[1]This instrument meets all current published specifications and has been calibrated using standards traceable to the National Institute of Standards and Technology (NIST) or other standards accepted by NIST in accordance with ISO 10012-1.

TABLE 1. Body Weight, *Tibialis anterior* (TA) Weight, and Area of Muscle Fibers[a]

Groups	Initial body weight (g)	Final body weight (g)	Laser irradiated TA weight (g)	Laser irradiated TA area (μm^2)	Sham TA weight (g)	Sham TA area (μm^2)
Daily dose of 3 J/cm^2 (n = 8)	40 ± 2	43 ± 4	0.1 ± 0.01	2915 ± 1704	0.1 ± 0.02	2555 ± 760
Daily dose of 10 J/cm^2 (n = 8)	41 ± 2	52 ± 3.5*	0.08 ± 0.01	4015 ± 890	0.09 ± 0.01	3762 ± 898

[a]Data are mean ± standard deviation.
*P = 0.002 (Student t-test), when compared to the average of final body weight.

referred with the understanding that it might also be comprised of type IIA, IIB, and IIX.

Muscle Morphology

The analysis of damaged fibers and the distribution of muscle type fibers (I, II, and IIC) were evaluated using toluidine blue-stains and m-ATPase- reacted sections, respectively, obtained from the middle region of the TA muscle. Serial cross-sections were evaluated by using a light microscope (Axiolab, Karl Zeiss, Germany) linked to a video printer images (Color Video Printer Mavigraph, Sony). The area of muscle fibers were measured in a PC computer using Vinspec software (software for morphological evaluation of muscle fibers, developed by the Group of Cybernetic Vision, Institute of Physic, Universidade de São Paulo, São Carlos, SP, Brazil). Muscle fibers were evaluated by one individual, who did not have previous access to the identification of each TA muscle (masked analysis).

Chronic signals of previous muscle fiber injury were identified by the presence of split fibers, small regenerated fibers, and centralized nuclei, without presence of acute signals of injury [32,36–38]. Acute signs of muscle tissue damage are usually identified by the presence of necrotic muscle fibers, phagocytosis, cellular infiltration, interstitial edema, an increase of ribosomal activity visible in the basophilic areas of muscle fibers, presence of a large centralized nucleus with a prominent nucleolus, hypercontracted myofibrils, and intracellular fragmentation [32,36,39].

Because regenerated TA muscles contained mainly type II fibers (type I and IIC were rarely observed), only muscle fiber area (μm^2) of type II fibers were measured in each TA muscle using a single cross-section obtained from the middle region of the muscle and submitted to mATPase reaction, in which type II fibers were identified. Hundred muscle fibers from each TA muscle were evaluated. The fibers, identified from a single histological cross-section, were randomly chosen by moving the coaxial drive for stage movement of the microscope, in the same direction, from the deep to the superficial region of the muscle section.

Statistic Analysis

Right and left TA muscles were randomly chosen to receive laser irradiation. Body and TA muscle weights and muscle fiber area were analyzed by Student t-test. Significant level accepted was 5%.

RESULTS

Body and *Tibialis Anterior* Weights

Although there was no difference in the initial average body weight of animals irradiated with 3 and 10 J/cm^2 (40 ± 2 g vs. 41 ±2 g, respectively), a significant difference was observed in the final body weight between the groups (43 ± 4 g vs. 52 ± 3.5 g, P = 0.002, respectively), i.e., animals irradiated with the dose of 10 J/cm^2 showed a significant gain of body weight (Table 1). Despite the difference observed in the final body weight, no differences were found between average muscle weight when laser irradiated and sham TA muscles of 3 J/cm^2 (0.1 ±0.01g vs. 0.1 ± 0.02 g, respectively) and 10 J/cm^2 groups (0.08 ± 0.02 g vs. 0.09 ± 0.01g, respectively) were compared (Table 1).

Morphological Aspect of Muscle Fibers

Twenty one days after the injection of ACL myotoxin, there were no qualitative differences in the morphological pattern of regenerated TA muscle fibers when both laser irradiated (3 and 10 J/cm^2) and sham muscles were analyzed by light microscope (Fig. 1). Histological cross-sections of TA muscles showed intensive presence of fibers with one or more centralized nucleus and small regenerated muscle fibers in all regions evaluated (Fig. 1). No fibrosis were observed in both laser irradiated and sham regenerated muscles.

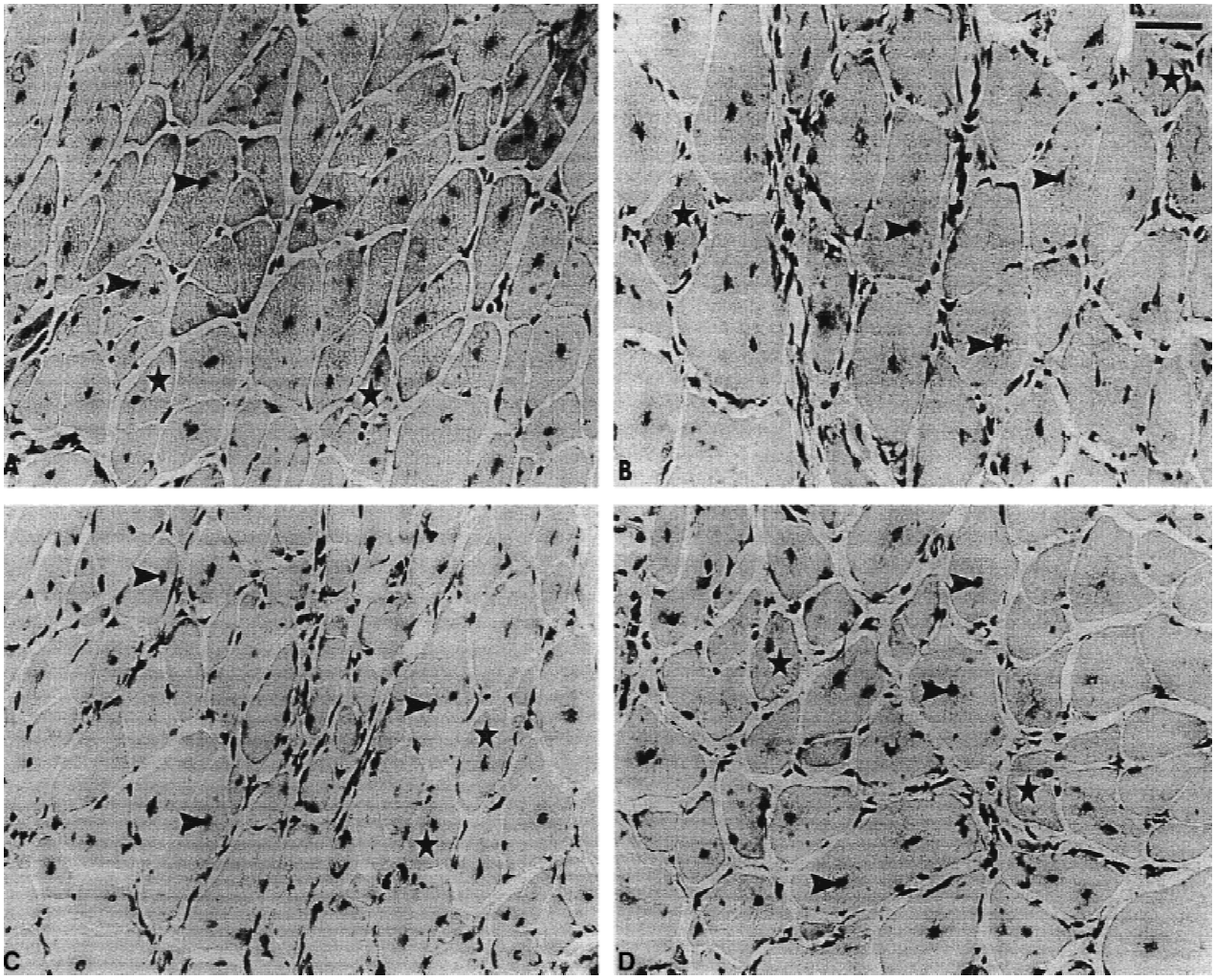

Fig. 1. Toluidine Blue staining. Cross-sections of *Tibialis anterior* muscle of mouse, 21 days after injury induced by ACL myotoxin. Regenerated muscles of both laser irradiated and sham groups showed a similar morphological pattern. Note that laser irradiated (**A:** 3 J/cm^2; **C:** 10 J/cm^2) and regenerated muscles of sham groups (**B:** sham of 3 J/cm^2 group; **D:** sham of 10 J/cm^2 group) presented small regenerated fibers (star) and fibers with centralized nucleus (arrowhead). Scale bar: 31 μm.

Some small regenerated muscle fibers showed positive reaction for AChE, which indicate that they are probably enervated (Fig. 2). The regenerated regions of TA muscle showed exclusive presence of type II fibers (Fig. 2). Type I fibers were rarely identified and were observed only in the normal bundles of the muscle. Type IIC were also rarely observed in the regenerated regions of the TA muscle.

Muscle Fiber Area

There was no difference in the average of muscle fiber area between GaAs laser irradiated and sham TA muscles in both doses of 3 J/cm^2 (2,915 ± 1,375 μm^2 vs. 2,555 ± 760 μm^2, respectively) and 10 J/cm^2 (4,015 ± 890 vs. 3,762 ± 898 μm^2, respectively) used (Table 1). It was interesting to note that in general, both irradiated and sham TA muscles of 10 J/cm^2 group showed an increased average area (3,888 μm^2) when compared with 3 J/cm^2 group (2,735 μm^2), although this difference was not significant.

To better evaluate the effect of 3 and 10 J/cm^2 doses of GaAs laser in the area of TA muscle fibers the cross-sectional area of muscle fibers among 0–4,000 μm^2 was analyzed (Fig. 3), and no significant differences between laser irradiated and sham muscles were found.

DISCUSSION

The results of this work indicate that doses of 3 and 10 J/cm^2 of GaAs laser, applied on the

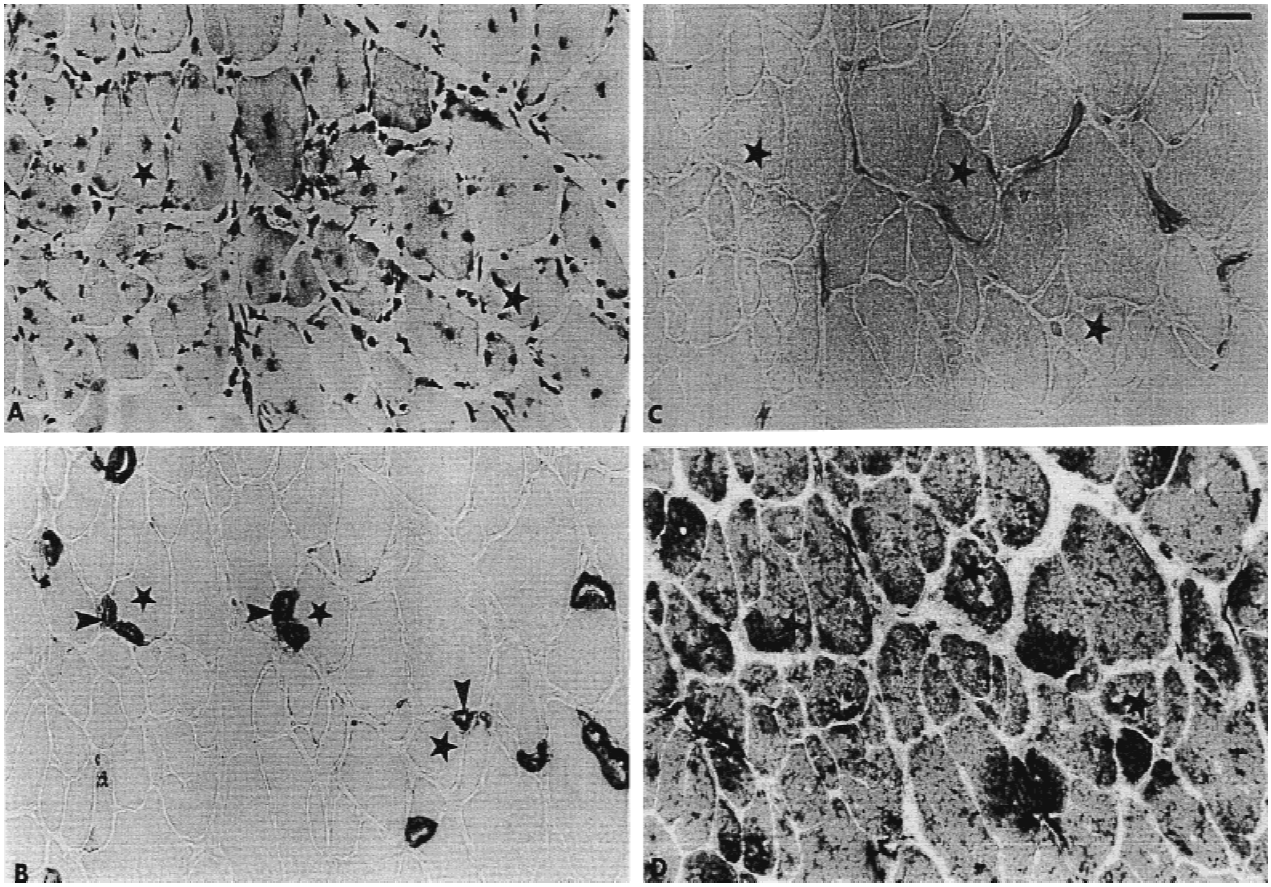

Fig. 2. Serial cross-sections of *Tibialis anterior* muscle of mouse, 21 days after injury induced by ACL myotoxin, and treated with GaAs laser (10 J/cm^2). **A:** Toluidine Blue staining. Note that some regenerated fibers (star) showed positive AChE reaction (**B:** arrowhead), which suggest that they are innervated. Myosin ATPase reactions (**C:** ac-mATPase, pH 4.3; **D:** alc-mATPase pH 10.3) show a regenerated region with exclusive presence of muscle type II fibers. Scale bar: 31 μm.

intact skin of injured TA muscle of mouse, were not effective to produce significant morphological changes in the regenerated muscle. Previous study developed in our laboratory to investigate the effect of HeNe laser in the regeneration process of mouse skeletal muscle, using the same protocol as described here, demonstrated that among different doses of HeNe laser, only the doses of 2.6 J/cm^2 promoted significant increase in the mitochondrial density and also in the area of muscle fibers (Amaral, Parizzotto, and Salvini, unpublished results). The comparison between these two studies, which were developed using the same species of animal, similar protocol to induce muscle injury and to apply the laser irradiation, but different types and doses of low power lasers, suggests that when irradiated on the skin, low doses of HeNe laser are more efficient to promote regeneration in injured skeletal muscles of mouse than low doses of GaAs laser.

These results indicate that despite the general acceptance that infrared lasers are more efficient in the treatment of deeper tissues than red lasers [1,2], HeNe laser irradiation was more effective than GaAs laser to promote skeletal muscle regeneration in mouse. The mechanism involved in this process is not known. It is possible that the muscle fibers do not have specific chromophore absorption for the wavelength of GaAs laser irradiation used. It is also possible that some morphological changes occurred in the first days of the regeneration process, which could not be observed 21 days after induced muscle injury. Also, some questions remain to be answered as for example the role of the inflammatory process induced by the myotoxin in the penetration and absorption of HeNe and GaAs lasers.

Studies in vitro to evaluate the skin transmittance of low power laser showed that the penetration of both HeNe and infrared lasers were for only a few millimeters, and the most important absorption was observed at the depth level of 0.4

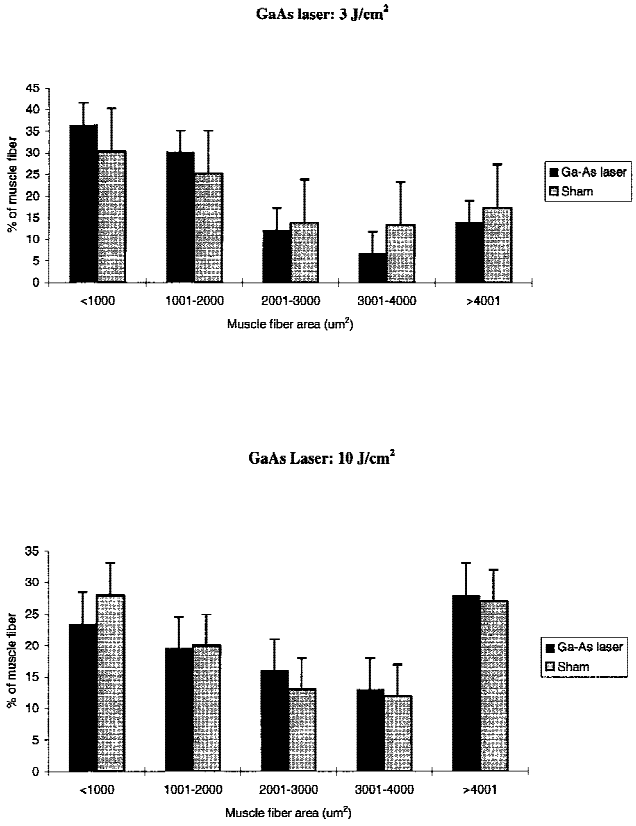

Fig. 3. Average area (μm^2) of *Tibialis anterior* muscle fibers, in both GaAs laser irradiated (■) and sham nonirradiated (□) groups, 21 days after muscle injury induced by ACL myotoxin.

and 0.5 mm [40]. The authors suggested that the laser therapy did not have really direct effects on deep tissues and probably the effects can be mediated by many different pathways.

It has been suggested that laser radiation has a systemic effect [1,24,25]. Some effects on the immune system has also been described such as sedimentation rates, platelet aggregation, leukocyte counts, and C reactive protein concentration [41,42,43].

A possible systemic effect could explain the significant gain of body weight observed in the animals irradiated with a dose of 10 J/cm^2. It was interesting to note that despite the significant difference in the body weight between the groups there was no difference in the muscle weight, which indicate that there was not a specific action of laser irradiation on the gain of TA muscle weight. This result is important because the spot size relative to the muscle size was probably similar between laser irradiated and sham muscles.

Although not statistically significant, the average of muscle fiber area in both irradiated and sham muscles of animals irradiated with 10 J/cm^2 was also increased. Maybe the small number of animals used limited the statistical evaluation and masked this result.

Previous reports demonstrated that ACL myotoxin is an excellent model to induce damage in both type I and type II fibers of mouse skeletal muscles with rearrangement of the motor units and changes in the incidence of muscle type fibers [38,39]. The predominant presence of regenerated type II fibers observed in the TA muscles was also found in the superficial region of gastrocnemius muscle of mouse 21 days [39] and 8 months [38] after muscle damage induced by ACL myotoxin. TA muscle is predominantly composed (90%) by type II fibers [44].

The presence of split fibers in regions where muscle injury usually occurred is considered a consequence of imperfect muscle fiber regeneration. Their presence is an evidence of the rearrangement of motor units after injury [36,45]. The presence of AChE activity in the split fibers and in the small regenerated fibers observed here 21 days after muscle injury, in both laser irradiated and sham regenerated TA muscles, associated with the absence of type I and exclusive presence of type II and IIC fibers in the regenerated regions of the muscle, suggest that the injury produced by ACL myotoxin induced axonal sprouts and rearrangement of the motor units, which determined type II muscle fibers. The probable mechanism for the rearrangement of motor units after injury is denervation of the necrotized muscle fibers and subsequent reinervation of regenerated muscle fibers by axonal sprouting [36,45,46].

Most of in vivo or in vitro studies, which described the beneficial effect of low-power energy lasers to promote healing or regeneration of different biological tissues, used direct tissue irradiation. Also, studies relating to skeletal muscle regeneration showed beneficial effects when the muscle were directly irradiated. Direct irradiation of HeNe laser utilized in the first 5 days after partial excision of the rat *gastrocnemius* promoted muscle maturation in the injured zone, which was observed by quantitative histological morphometric methods [4]. Similar results were also found in amphibian *gastrocnemius* muscles, where direct laser irradiation stimulated the volume fraction of the myotubes and increased the incidence of young myofibers in the regenerated muscles [6]. Recently it was described that GaAs laser (2.82 Hz, 0.005 mW), applied during the 14 days following cold injury to the toad gastrocne-

mius muscle, improve muscle regeneration in a similar way than HeNe laser [7].

Unfortunately, the effect of low-power lasers on the muscle injury and muscle regeneration process has not been sufficiently studied, specially using similar models of muscle injury as observed in humans and frequently submitted to conservative treatments.

In conclusion, the results of this study indicate that doses of 3 and 10 J/cm^2 of GaAs laser, applied to the intact skin of injured muscles, are not efficient to promote significant morphological changes in the regenerated skeletal muscle of mouse, but the dose of 10 J/cm^2 promoted significant gain of body weight.

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